

Characterization of a Plasma Membrane Calcium Oscillator in Rat Pituitary Somatotrophs*

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In excitable cells, oscillations in intracellular free calcium concentrations ($[Ca^{2+}]_i$) can arise from action-potential-driven Ca^{2+} influx, and such signals can have either a localized or global form, depending on the coupling of voltage-gated Ca^{2+} influx to intracellular Ca^{2+} release pathway. Here we show that rat pituitary somatotrophs generate spontaneous $[Ca^{2+}]_i$ oscillations, which rise from fluctuations in the influx of external Ca^{2+} and propagate within the cytoplasm and nucleus. The addition of caffeine and ryanodine, modulators of ryanodine-receptor channels, and the depletion of intracellular Ca^{2+} stores by thapsigargin and ionomycin did not affect the global nature of spontaneous $[Ca^{2+}]_i$ signals. Bay K 8644, an L-type Ca^{2+} channel agonist, initiated $[Ca^{2+}]_i$ signaling in quiescent cells, increased the amplitude of $[Ca^{2+}]_i$ spikes in spontaneously active cells, and stimulated growth hormone secretion in perfused pituitary cells. Nifedipine, a blocker of L-type Ca^{2+} channels, decreased the amplitude of spikes and basal growth hormone secretion, whereas Ni^{2+} , a blocker of T-type Ca^{2+} channels, abolished spontaneous $[Ca^{2+}]_i$ oscillations. Spiking was also abolished by the removal of extracellular Na^+ and by the addition of 10 mM Ca^{2+} , Mg^{2+} , or Sr^{2+} , the blockers of cyclic nucleotide-gated channels. Reverse transcriptase-polymerase chain reaction and Southern blot analyses indicated the expression of mRNAs for these channels in mixed pituitary cells and purified somatotrophs. Growth hormone-releasing hormone, an agonist that stimulated cAMP and cGMP productions in a dose-dependent manner, initiated spiking in quiescent cells and increased the frequency of spiking in spontaneously active cells. These results indicate that in somatotrophs a cyclic nucleotide-controlled plasma membrane Ca^{2+} oscillator is capable of generating global Ca^{2+} signals spontaneously and in response to agonist stimulation. The Ca^{2+} -signaling activity of this oscillator is dependent on voltage-gated Ca^{2+} influx but not on Ca^{2+} release from intracellular stores.

The immortalized GH,¹ GS, AtT-20, and α T3 pituitary cells have been frequently used as cell models for the characteriza-

tion of electrical activity and the associated Ca^{2+} signaling in lactotrophs, somatotrophs, corticotrophs, and gonadotrophs, respectively (1–10). Simultaneous measurements of electrical activity and intracellular free Ca^{2+} concentrations ($[Ca^{2+}]_i$) in GH₃B₆ pituitary cells confirmed the dependence of $[Ca^{2+}]_i$ on action potential (AP) firing (1). These cells express T-type and L-type voltage-gated Ca^{2+} channels (VGCCs), tetrodotoxin (TTX)-sensitive Na^+ channels, and several voltage-gated and Ca^{2+} -controlled K^+ channels, which are all involved in the spontaneous firing of APs (11). Briefly, TTX-sensitive Na^+ channels, T-type Ca^{2+} channels, and dihydropyridine-sensitive L-type Ca^{2+} channels control the slow pacemaker depolarization and drive the rapid depolarizing phase of APs. On the other hand, several K^+ channels control the repolarization phase of APs, including delayed rectifier, BK, and SK channels (11–13). The transient opening of VGCCs may provide a window for the coupling of electrical activity to calcium-induced calcium release (CICR) through ryanodine receptor channels (RyR) (7). In line with this, a caffeine-sensitive intracellular Ca^{2+} pool (14) and the cADP ribose-dependent signaling pathway were identified in these cells (15). Thus, the plasma membrane oscillator in immortalized pituitary cells resembles the one that is operative in cardiac and some neuronal cells (16–18).

At the present time, however, it is not clear to what extent the native anterior pituitary cells differ from the immortalized cells. For example, Kwiecien *et al.* (19) reported that cultured somatotrophs behave only as “conditional pacemakers.” In other words, these cells are quiescent, and the addition of growth hormone-releasing hormone (GHRH) is required to initiate firing. This is in contrast with the results by Sims *et al.* (20) and Holl *et al.* (21), who observed spontaneous firing of APs and extracellular Ca^{2+} -dependent fluctuation in $[Ca^{2+}]_i$ in a fraction of somatotrophs. Several laboratories have also reported that gonadotrophs, lactotrophs, and corticotrophs exhibit periods of spontaneous firing of APs (22–25). In gonadotrophs, voltage-gated Ca^{2+} influx forms membrane-localized $[Ca^{2+}]_i$ signals, which are not sufficient to trigger gonadotropin secretion (13). Basal ACTH, TSH, and FSH secretion are also low and are unaffected by the removal of extracellular Ca^{2+} . In contrast, basal GH and prolactin secretions are high and controlled by spontaneous Ca^{2+} influx through L-type Ca^{2+} channels (26). Since all these cells exhibit spontaneous firing of APs (11), these observations raised the possibility that AP-driven Ca^{2+} signals in somatotrophs and lactotrophs, but not in other pituitary cells, are coupled to CICR, leading to the generation of global $[Ca^{2+}]_i$ signals sufficient to trigger exocytosis. Here we studied the nature of Ca^{2+} influx-dependent $[Ca^{2+}]_i$ tran-

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¹ The abbreviations used are: GH, growth hormone; APs, action potentials; VGCCs, voltage-gated calcium channels; CNGs, cyclic nucleotide-gated channels; RyR, ryanodine receptor-channels; TTX, tetrodotoxin; $[Ca^{2+}]_i$, intracellular calcium concentration; GHRH, growth hormone-releasing hormone; CICR, calcium-induced calcium release;

NMDG, N-methyl-D-glucamine; RT-PCR, reverse transcriptase-polymerase chain reaction.

sients in somatotrophs and their relevance in growth hormone secretion.

MATERIALS AND METHODS

Cell Cultures and Hormone Secretion—Anterior pituitary glands from adult female Harlan Sprague-Dawley rats were obtained from Charles River Inc. (Wilmington, MA) and were dispersed into single cells by a trypsin/DNase (Sigma) treatment procedure as described previously (23). All experiments were performed in either identified cells from mixed pituitary cell populations or purified somatotrophs. The $[Ca^{2+}]_i$ responses to GnRH, TRH, and GHRH, applied at the end of experiments, were used for identification of cells from a mixed population. Cell purification was done by a Percoll discontinuous density gradient centrifugation (27).

For cell column perfusion, 12×10^6 mixed cells were incubated with preswollen cytochrome-1 beads (Amersham Pharmacia Biotech) in 60-mm culture dishes for 2 days. The cells were then loaded into temperature-controlled chambers and perfused at 37 °C with Hanks' M199 containing 20 mM HEPES and 0.1% bovine serum albumin for 60 min at a flow rate of 0.8 ml/min for 2 h, after which a stable basal secretion rate was established. During the test period, 1-min fractions were collected, and the perfusate was subsequently stored at -20 °C.

For cAMP and cGMP measurements, 1.0×10^6 cells per well were cultured in 24-well plates for 24 h. Cultures were washed and stimulated with GHRH in the phosphodiesterase-free Hanks' M199 for 30 min. GH, cAMP, and cGMP contents were determined by radioimmunoassay using the reagents and standards provided by Dr. Parlow and the National Hormone and Pituitary Program (Torrance, CA), Antibodies Inc. (Davis, CA), and Albert Baukal (NIH, Bethesda).

Calcium Imaging Measurements—Cells were plated on coverslips coated with poly-L-lysine and cultured in medium 199 containing Earle's salts, sodium bicarbonate, horse serum, and antibiotics for 16–48 h. Both mixed populations of anterior pituitary cells and highly purified somatotrophs were employed for $[Ca^{2+}]_i$ measurements. Cells were incubated for 60 min at 37 °C with 2 μ M fura-2 AM (Molecular Probes, Eugene, OR) in phenol red-free medium 199 containing Hanks' salts, 20 mM sodium bicarbonate, and 20 mM HEPES, pH 7.4. Coverslips with cells were washed with phenol red-free Krebs-Ringer buffer and mounted on the stage of an Axiovert 135 microscope (Carl Zeiss, Oberkochen, Germany) attached to an Attolfluor Digital Fluorescence Microscopy System (Atto Instruments, Rockville, MD). In experiments without external Na^+ , this cation was replaced with 120 mM *N*-methyl-D-glucamine (NMDG). Cells were examined under a $\times 40$ oil immersion objective during exposure to alternating 340 and 380 nm light beams, and the intensity of light emission at 505 nm was measured. $[Ca^{2+}]_i$ is shown as the ratio of intensities measured at 340 and 380 nm $[F_{340}/F_{380}]$.

The intracellular distribution of Ca^{2+} signals was examined by laser scanning confocal microscopy. Purified somatotrophs were kept for 30 min at 37 °C and for additional 30 min at room temperature in medium M199 with Hanks' salts and 4 μ M fluo-3 AM (Molecular Probes). The solution was then replaced with Krebs-Ringer buffer with 2 mM $CaCl_2$. Coverslips with cells were mounted on a stage of an inverted microscope (Nikon Diaphot 300) attached to a Bio-Rad MRC 1024 system (Bio-Rad). Laser line of 514 nm was used for the excitation, and the emitted light was collected at 540 nm. Laser power was reduced to 1% in order to enable repetitive scanning without damaging the cell. Images were collected under $\times 40$ oil immersion objective and further zoom ($\times 2$ and 2.5) was also applied. Data acquisition was controlled by LaserSharp, version 3.2 time course software (Bio-Rad). The time period between two points in planar scans (XY scans) was about 1.5 s, and 6–7 ms for line scans (XT scans). Each series of line scans lasted for 6.4 s. Data are presented as increase in basal fluorescence (F/F_0).

RT-PCR Analysis of Cyclic Nucleotide-gated Channels (CNGs)—Total RNA was isolated from mixed and purified populations of pituitary primary cell cultures using TRIZOL™ reagent (Life Technologies, Inc.) and was treated with DNase I at 37 °C for 30 min. After heat inactivation of DNase I by incubating at 70 °C for 15 min, first strand cDNA was synthesized from 5 μ g of total RNA, using Superscript II reverse transcriptase and oligo(dT)_{12–18} primers (Life Technologies, Inc.) in a reaction volume of 20 μ l. Aliquot (1 μ l) of the resulting single-strand cDNA was used for the PCRs, which were performed in 25- μ l volume, containing 200 μ M each of four deoxynucleotide triphosphates, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 2 mM $MgCl_2$, and 0.5 units of Ex Taq polymerase (Panvera Corp., Madison, WI). Nucleotide sequences for the PCR primers correspond to cytoplasmic carboxyl regions of rat rod CNG1 (28), cone CNG2 (29), and olfactory CNG2 (30) and have the

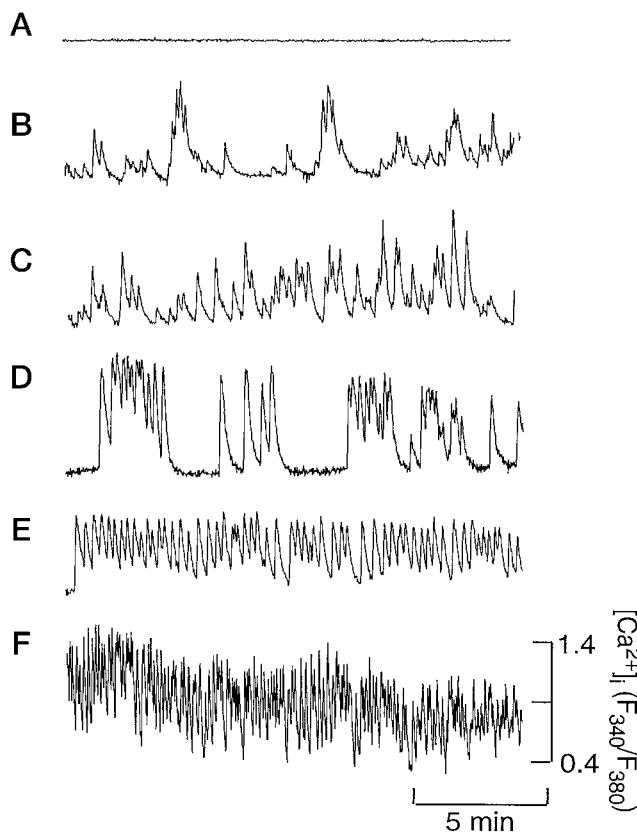


FIG. 1. Basal $[Ca^{2+}]_i$ in cultured somatotrophs. A, about 50% of somatotrophs were silent. B–F, pattern of Ca^{2+} signals in spontaneously active cells. The tracings shown are from identified somatotrophs from mixed populations of anterior pituitary cells. In this and the following figures, $[Ca^{2+}]_i$ tracings shown are representative of at least 20 recordings in three to ten independent experiments. Fura 2 was employed as a dye for $[Ca^{2+}]_i$ recordings, if not otherwise specified.

following sequences: CNG1U (sense), 5'-TGCGAATTTGGGCAGTG-ACC-3', CNG1L (antisense), 5'-TCTCCTCCGGGTCCTCAAG-3'; CNG2U (sense), 5'-AGGGCAGATGCCAGGAACAT-3', CNG2L (antisense), 5'-CGAGGCTGTAAAGTGCTCA-3'; CNG3U (sense), 5'-GGAGGTAGATGTTTCAGGAGA-3', and CNG3L (antisense), 5'-CTTTGGGAGAGTTCAGAGG-3'.

PCRs were run for 30 cycles at 94 °C, 1 min (denaturation); 55 °C, 35 s (annealing); 72 °C, 1 min (extension), followed by a final extension for 10 min at 72 °C. Amplified DNA fragments were electrophoresed on 1% agarose gel and visualized with ethidium bromide. The same volumes of samples used for CNG mRNA analysis were also subjected to PCR using glyceraldehyde-3-phosphate dehydrogenase-specific primers (31). PCR products separated on a gel were then subjected to Southern blot analysis. Oligonucleotide probe specific to rod CNG1 has the following sequence, 5'-CGAATCTTGGCTGAGTATGAATCGATGCAGCA-G-3'. The probe was labeled at the 5'-end with [γ -³²P]ATP (5000 Ci/mmol) using T4 polynucleotide kinase (PanVera Corp.). Pre-hybridization was performed at 65 °C for 2 h in 6 \times SSPE, 0.01 M sodium phosphate, pH 6.8, 1 mM EDTA, 0.5% SDS, 100 mg/ml salmon sperm DNA, and 0.1% dried milk. Hybridization was performed under the same conditions containing 0.5 pmol/ml radiolabeled probe for 3 h. Membranes were then washed at 42 °C for 10 min in 2 \times SSPE and at 65 °C for 10 min and exposed to x-ray film (Eastman Kodak Co.).

RESULTS

Extracellular Calcium Dependence of Spontaneous $[Ca^{2+}]_i$ Transients—About 50% of somatotrophs, examined 16–48 h after dispersion, exhibited no changes in basal $[Ca^{2+}]_i$, and these cells are further referred to as quiescent (Fig. 1A). The residual cells showed fluctuations in $[Ca^{2+}]_i$ and are referred to as spontaneously active cells. As shown in Fig. 1, B–F, the pattern of spontaneous $[Ca^{2+}]_i$ oscillations was variable among the cells. Some somatotrophs exhibited prolonged bursts of elevated $[Ca^{2+}]_i$ (Fig. 1, B–D), and others exhibited shorter

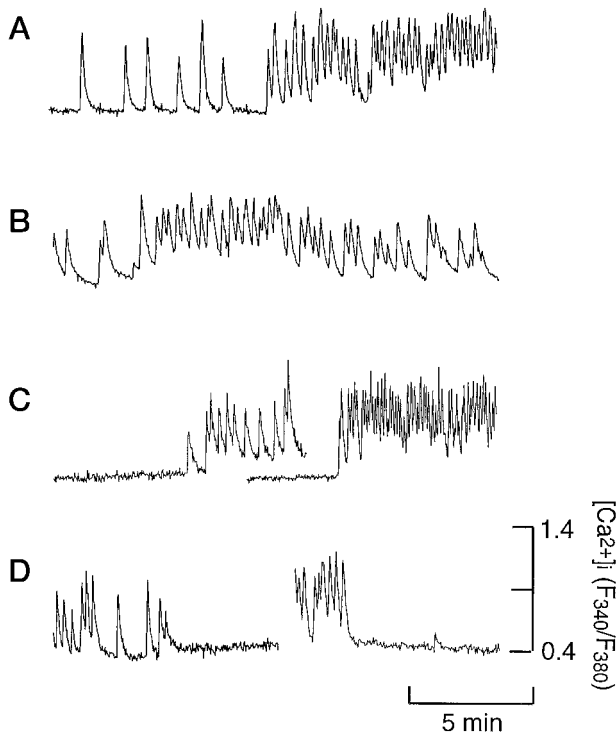


FIG. 2. The rhythm of Ca^{2+} signaling in cultured somatotrophs. A and B, spontaneous modulation of frequency of $[\text{Ca}^{2+}]_i$ transients. C and D, transitions from quiescent to active status (Fig. 2C) and from active to quiescent status (Fig. 2D).

base-line-like $[\text{Ca}^{2+}]_i$ transients (Fig. 1, E and F). In spontaneously active cells, a transition from low frequency (0.5–2 spikes per min) to higher frequency (3–10 spikes/min) of $[\text{Ca}^{2+}]_i$ transients, and vice versa, was frequently observed (Fig. 2, A and B). Transitions from quiescent to active status (Fig. 2C) and from active to quiescent status (Fig. 2D) were also observed.

Spontaneous $[\text{Ca}^{2+}]_i$ transients were abolished by the addition of 2 mM EGTA-containing medium, indicating a role for Ca^{2+} influx in their generation (Fig. 3A, top tracing). Removal of extracellular Ca^{2+} did not affect base-line $[\text{Ca}^{2+}]_i$ in quiescent cells (Fig. 3A, bottom tracing). Depolarization of cells with 50 mM K^+ also abolished $[\text{Ca}^{2+}]_i$ oscillations (Fig. 3B, top tracing). In quiescent and spontaneously active cells, 50 mM K^+ induced a peak in the $[\text{Ca}^{2+}]_i$ response of comparable amplitude (Fig. 3B). The response to K^+ was about twice as large as the peak of spontaneous $[\text{Ca}^{2+}]_i$ transients (Table I). The average peak $[\text{Ca}^{2+}]_i$ of spontaneous transients in somatotrophs was about 75% that observed in $[\text{Ca}^{2+}]_i$ response to the calcium-mobilizing agonist for these cells, endothelin-1 (Table I). Thus, a plasma membrane Ca^{2+} oscillator is operative in cultured somatotrophs and drives relatively high amplitude $[\text{Ca}^{2+}]_i$ transients.

Spontaneous $[\text{Ca}^{2+}]_i$ transients were not unique to somatotrophs, as they were also observed in other pituitary cells, including lactotrophs, and gonadotrophs. Spontaneous $[\text{Ca}^{2+}]_i$ transients were observed in about 40 and 20% of lactotrophs and gonadotrophs, respectively. In the same preparation, the amplitudes of spontaneous $[\text{Ca}^{2+}]_i$ transients in somatotrophs were significantly higher than those observed in the other two pituitary cell types analyzed (Table I). In contrast, the amplitudes of K^+ -induced $[\text{Ca}^{2+}]_i$ responses were comparable among these cells (Table I), indicating that somatotrophs, lactotrophs, and gonadotrophs do not differ with respect to their voltage-gated Ca^{2+} influx capacity.

Role of VGCCs in Spontaneous $[\text{Ca}^{2+}]_i$ Transients and GH Release—To examine the role of VGCCs in spontaneous $[\text{Ca}^{2+}]_i$

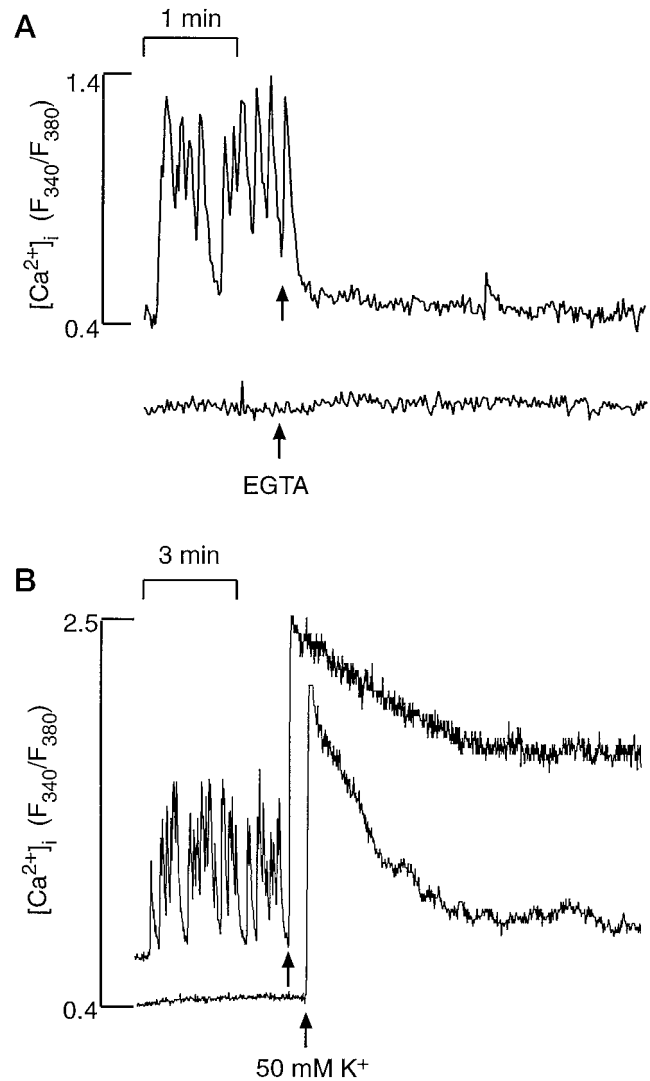


FIG. 3. Characterization of spontaneous $[\text{Ca}^{2+}]_i$ transients. A, extracellular Ca^{2+} dependence of spontaneous $[\text{Ca}^{2+}]_i$ transients. Cells were initially bathed in 2 mM Ca^{2+} -containing medium and subsequently in Ca^{2+} -deficient medium (free $[\text{Ca}^{2+}]$ about 100 nM), adjusted by EGTA. Bottom tracing illustrates the lack of effects of extracellular Ca^{2+} depletion on $[\text{Ca}^{2+}]_i$ in quiescent cells. B, depolarization-induced $[\text{Ca}^{2+}]_i$ response in spontaneously active and quiescent somatotrophs. In this and the following figures, arrows indicate the moment of drug applications. The drugs were added in 1-ml volumes and were present throughout the recording. The concentrations indicated above the arrows are final.

TABLE I
Comparison of the amplitudes of spontaneous, agonist-induced, and potassium-induced $[\text{Ca}^{2+}]_i$ responses in pituitary cells

Cell type	Spontaneous $[\text{Ca}^{2+}]_i$	Agonist-induced $[\text{Ca}^{2+}]_i^a$	Potassium-induced $[\text{Ca}^{2+}]_i$
Somatotrophs	1.23 ± 0.05^b (51)	1.66 ± 0.11 (34)	2.21 ± 0.40 (42)
Lactotrophs	0.94 ± 0.03 (38)	1.40 ± 0.11 (52)	2.02 ± 0.10 (17)
Gonadotrophs	0.90 ± 0.10 (10)	1.86 ± 0.10 (22)	2.22 ± 0.18 (16)

The results shown are means \pm S.E. of F_{340}/F_{380} .

^a Somatotrophs were stimulated with endothelin-1, lactotrophs with TRH, and gonadotrophs with GnRH, all applied at 100 nM concentrations.

^b $p < 0.05$ compared with spontaneous $[\text{Ca}^{2+}]_i$ in lactotrophs and gonadotrophs.

transients in somatotrophs, dihydropyridines, Cd^{2+} , and Ni^{2+} were employed. Bay K 8644, an L-type calcium channel agonist, was effective in relatively high concentrations (Fig. 4). In silent somatotrophs, 1 μM Bay K 8644 induced $[\text{Ca}^{2+}]_i$ tran-

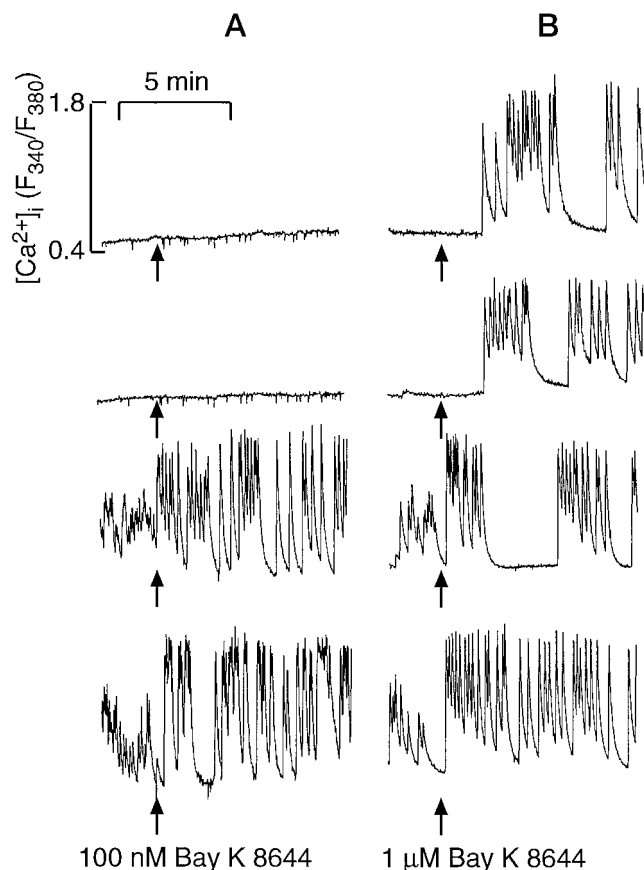


FIG. 4. Effects of Bay K 8644, an L-type Ca^{2+} channel agonist, on Ca^{2+} signaling in somatotrophs. Initiation of $[\text{Ca}^{2+}]_i$ transients by $1 \mu\text{M}$ Bay K 8644 (B, two top tracings) but not by 100 nM Bay K 8644 (A, two top tracings) in quiescent cells. Modulation of the amplitude of $[\text{Ca}^{2+}]_i$ transients in spontaneously active cells (A and B, two bottom tracings).

sients, whose pattern was highly comparable to that observed in spontaneously active cells (Fig. 4B, two top tracings). In a majority of oscillatory cells, $1 \mu\text{M}$ Bay K 8644 increased the amplitude of $[\text{Ca}^{2+}]_i$ transients (Fig. 4B, two bottom tracings). Some somatotrophs also responded to 100 nM Bay K 8644 by modulating the frequency of spontaneous $[\text{Ca}^{2+}]_i$ transients (Fig. 4A, two bottom tracings), whereas this concentration was ineffective in initiating $[\text{Ca}^{2+}]_i$ transients in a majority of quiescent cells (Fig. 4A, two top tracings). In all examined cells, 10 nM Bay K 8644 was ineffective (not shown).

Like Bay K 8644, nifedipine, an L-type calcium channel blocker, affected spontaneous $[\text{Ca}^{2+}]_i$ transients only in high concentrations. In about 50% of cells, $1 \mu\text{M}$ nifedipine abolished Ca^{2+} spiking, whereas in the residual cells it either had no effect or it changed the frequency and/or the amplitude of $[\text{Ca}^{2+}]_i$ transients (Fig. 5A). In a majority of cells, 100 nM nifedipine was ineffective or reduced the frequency of $[\text{Ca}^{2+}]_i$ transients (Fig. 5A). Addition of Cd^{2+} , a nonselective blocker of VGCCs, abolished $[\text{Ca}^{2+}]_i$ transients, but only in a small fraction of the cells, and changed the pattern of spiking in a majority of somatotrophs (Fig. 5B). In contrast, $100 \mu\text{M}$ Ni^{2+} , a relatively specific inhibitor of T-type Ca^{2+} channels, inhibited spontaneous and Bay K 8644-induced $[\text{Ca}^{2+}]_i$ transients in a majority of the cells (Fig. 5C).

The effects of dihydropyridines and Cd^{2+} on GH secretion were evaluated in perfused pituitary cells. The addition of $1 \mu\text{M}$ nifedipine reduced basal GH secretion to about 50% that observed in controls (Fig. 6A). In contrast, $1 \mu\text{M}$ Bay K 8644 increased GH secretion in a pulsatile-like manner (Fig. 6A). At

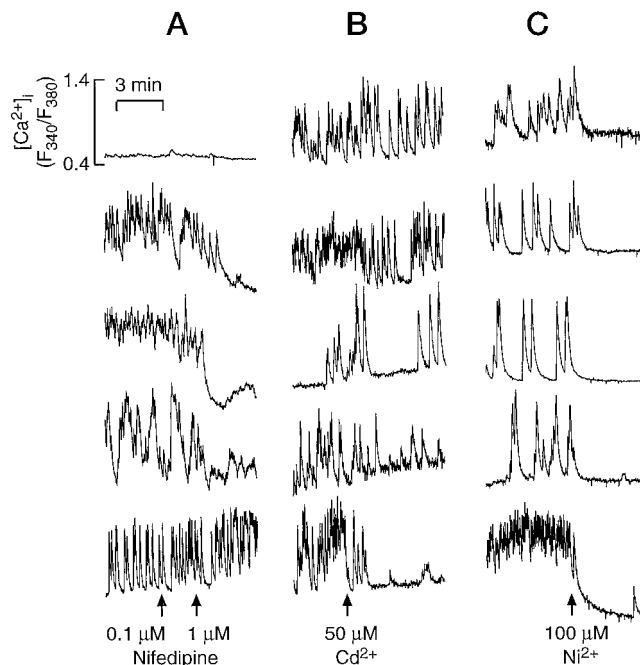


FIG. 5. Effects of voltage-gated calcium channel blockers on spontaneous $[\text{Ca}^{2+}]_i$ transients. A, concentration-dependent effects of nifedipine, an L-type Ca^{2+} channel antagonist, on the pattern of $[\text{Ca}^{2+}]_i$ transients. B, effects of $50 \mu\text{M}$ Cd^{2+} , a non-selective blocker of voltage-gated Ca^{2+} channels, on the pattern of $[\text{Ca}^{2+}]_i$ transients. C, effects of $100 \mu\text{M}$ Ni^{2+} , a relatively specific blocker of T-type Ca^{2+} channels, on spontaneous (three top tracings) and Bay K 8644 ($1 \mu\text{M}$)-induced $[\text{Ca}^{2+}]_i$ transients (two bottom tracings). The arrows at the bottom indicate the moments of drug application in all tracings shown above.

100 nM concentrations, nifedipine and Bay K 8644 were practically ineffective (Fig. 6B), and $50 \mu\text{M}$ Cd^{2+} had only a minor inhibitory effect (Fig. 6C).

Spatio-temporal Aspect of Spontaneous $[\text{Ca}^{2+}]_i$ Transients in Somatotrophs—To characterize the spatio-temporal aspects of Ca^{2+} signals in somatotrophs, $[\text{Ca}^{2+}]_i$ imaging was done by laser scanning confocal microscopy. For these measurements, highly purified somatotrophs were loaded with a Ca^{2+} indicator, fluo-3. Fig. 7 shows the temporal and spatial characteristics of Ca^{2+} signals measured simultaneously in two spontaneously active somatotrophs, stimulated with Bay K 8644 and subsequently by ionomycin. Measurements at the middle plane of these somatotrophs revealed that $[\text{Ca}^{2+}]_i$ rose in all cellular regions analyzed during spontaneous transients (Fig. 7, upper panel). In the somatotroph schematically shown on left top panel (cell-I), there was no obvious difference in the amplitude of $[\text{Ca}^{2+}]_i$ transients in three different regions of the cytoplasm (boxes and tracings 1, 2, and 4). A significant increase in $[\text{Ca}^{2+}]_i$ was also observed in the nucleoplasm, with the amplitude of response higher than that observed in cytoplasm (box and tracing 3).

Addition of Bay K 8644 increased the amplitude of $[\text{Ca}^{2+}]_i$ responses in all analyzed cell regions. The $[\text{Ca}^{2+}]_i$ profiles were highly comparable among the tracings and specific for each cell (tracings 1–4 of cell-I versus tracing 5 of cell-II). Ionomycin-induced Ca^{2+} release was also detected in all cellular regions analyzed, with the amplitude of $[\text{Ca}^{2+}]_i$ response higher than that observed during Bay K 8644 stimulation. During continuous stimulation with ionomycin, a recovery of the oscillatory response was observed in both cells but at different time points. The bottom panel in Fig. 7 shows the central plane images for cell-I at four different time points, indicated by arrows above trace 3 in the upper panel. Images demonstrate the global

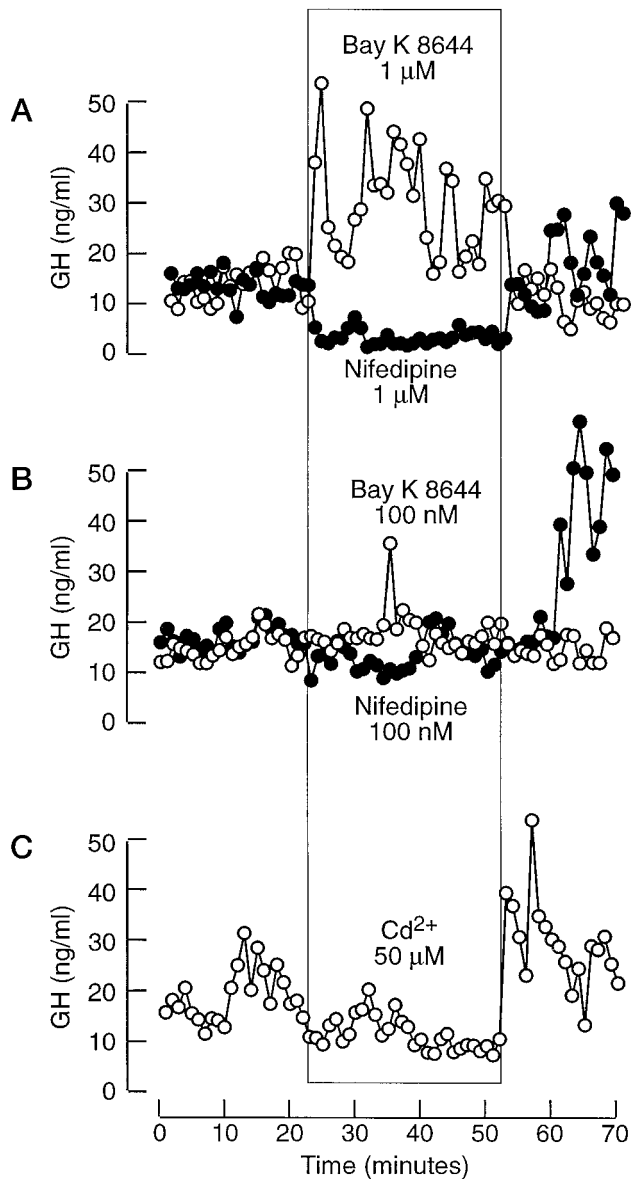


FIG. 6. Modulation of basal GH secretion by calcium channel agonists and antagonists in perifused pituitary cells. *A* and *B*, effects of dihydropyridines on basal GH secretion. Open circles indicate the experiment with Bay K 8644 and closed circles experiment with nifedipine. *C*, effects of Cd^{2+} on basal GH secretion. The rectangle indicates duration of perfusion with Bay K 8644, nifedipine, or Cd^{2+} . Fractions were collected every minute.

nature of changes in cytoplasmic and nuclear $[\text{Ca}^{2+}]_i$ in a cell from base line (*A*) to one of the peaks of the spontaneous transients (*B*), one of the peaks of Bay K 8644-induced transients (*C*), and after ionomycin-induced discharge from the intracellular Ca^{2+} pool (*D*).

To characterize further the generation of spontaneous $[\text{Ca}^{2+}]_i$ transients, a line scan mode of confocal imaging was performed simultaneously in two cells during the transition from quiescent to active stage. In a schematic representation of these two cells shown in Fig. 8, the right-hand side of the middle panel, the vertical line indicates where the recordings were done. The XT images, labeled as *A–C*, show the spatial nature of $[\text{Ca}^{2+}]_i$ signals during initiation of a $[\text{Ca}^{2+}]_i$ transient (*A*), during the peak $[\text{Ca}^{2+}]_i$ response (*B*), and between the two $[\text{Ca}^{2+}]_i$ peaks (*C*). These images again demonstrate the global nature of spontaneous $[\text{Ca}^{2+}]_i$ transients in somatotrophs, with the rise and the fall in $[\text{Ca}^{2+}]_i$ being obvious in both cytoplas-

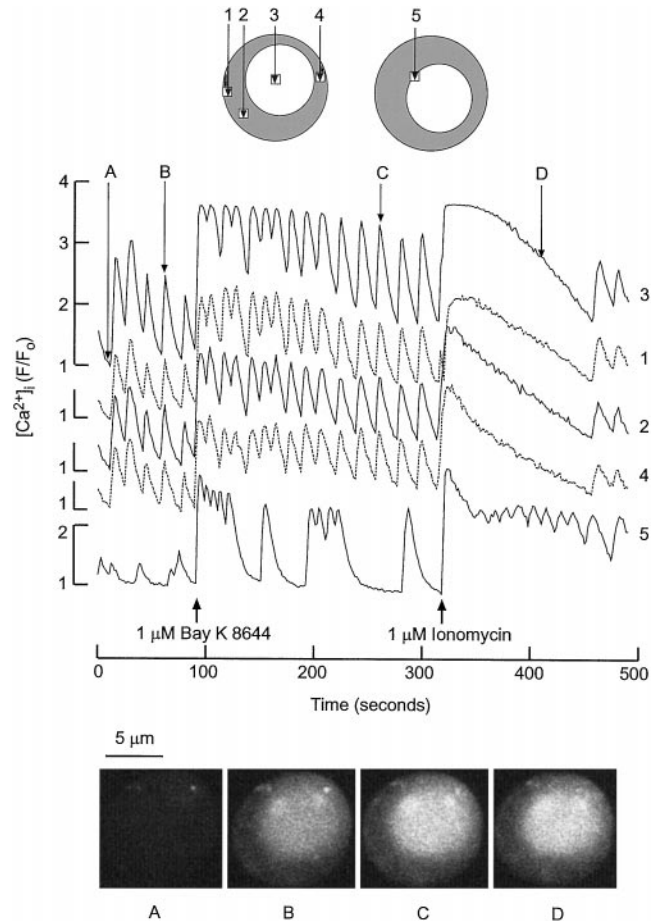


FIG. 7. Spatio-temporal distribution of spontaneous and Bay K 8644-induced $[\text{Ca}^{2+}]_i$ transients in purified somatotrophs. Confocal images of fluo-3-loaded cells were recorded and fluorescence intensities measured simultaneously in five different regions of two somatotrophs. As shown in the schematic representation above the plots, cell-I regions denoted by 1, 2, and 4 are in the cytoplasm and region 3 is in the nucleoplasm, whereas the cell-II region is denoted by 5 and covers a part of cytoplasm and a part of nucleoplasm. $[\text{Ca}^{2+}]_i$ profiles from these regions are presented as ratios between fluorescence intensities and basal fluorescence at the corresponding region. The numbers on the right correspond to the regions of interest shown above. Bottom panel shows images for cell-I at four different time points indicated by arrows above trace 3.

mic and nucleoplasmic compartments.

Fluorescence intensities were also recorded at three line segments, one in the sub-plasma membrane region (labeled as 3), another in the central region of cytoplasm (labeled as 1) and a third in the central region of nucleoplasm (labeled as 2). These data are presented as ratios between fluorescence intensity and the average base-line intensity for the corresponding line segments (F/F_0) in the upper panel of Fig. 8. As in XY scan shown in Fig. 7, the $[\text{Ca}^{2+}]_i$ profiles resembled each other in all monitored cell regions, suggesting that they were driven by the same mechanism. Also, the values of F/F_0 were still higher in the nucleoplasm (tracing 2 versus tracings 1 and 3). Finally, the extended time scale analysis of F/F_0 profiles shown in Fig. 8, upper panel, revealed that the rise in $[\text{Ca}^{2+}]_i$ occurred almost simultaneously in cytoplasmic and nucleoplasmic regions.

Independence of Spontaneous $[\text{Ca}^{2+}]_i$ Transients on Ca^{2+} Release from Intracellular Stores—The ability of somatotrophs to resume $[\text{Ca}^{2+}]_i$ oscillations after the depletion of intracellular Ca^{2+} pool by ionomycin (Fig. 7) argues against the coupling of electrical activity to Ca^{2+} release from intracellular stores. This conclusion was further supported in three types of experiments. First, the addition of 1 μM thapsigargin, a concentra-

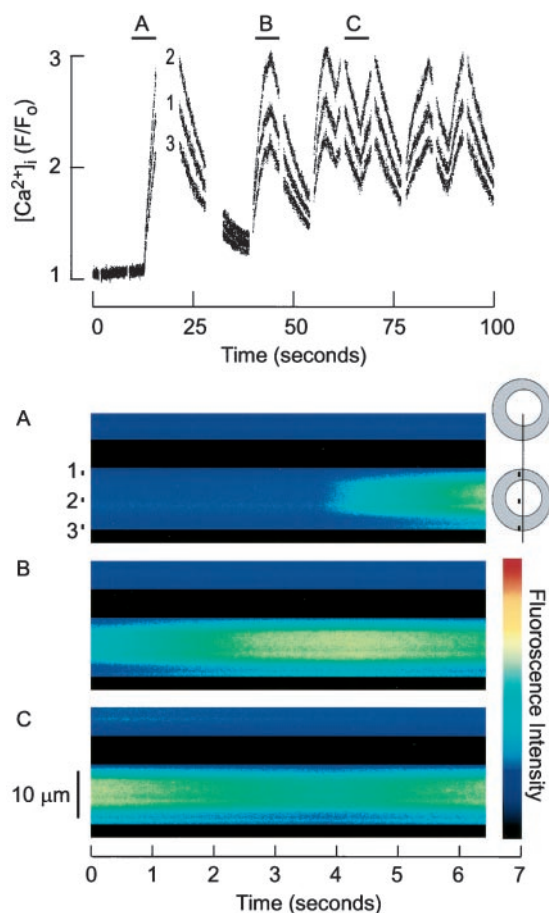


FIG. 8. Line scans of a somatotroph in transition from quiescent to active state. As shown in the schematic representation on the right, fluorescence was recorded along a line that passed through a diameter of one cell and partially through another cell. The line was scanned every 6–7 ms during 6.4 s; the XT image was saved and a new series of scans started. Fluorescence intensities were recorded at two line segments positioned in the cytoplasm (labeled as 1 and 3) and one positioned in the nucleoplasm (labeled as 2). Data are presented as ratios between fluorescence intensity at given time and the average base-line intensity for the corresponding line segments. The chosen images show the initiation of a $[\text{Ca}^{2+}]_i$ transient (A), a period before and after the peak (B), and a fall and subsequent rise in cytosolic and nuclear $[\text{Ca}^{2+}]_i$ (C). The horizontal lines above the $[\text{Ca}^{2+}]_i$ profiles indicate the time intervals at which presented XT images were recorded.

tion sufficient to block completely Ca^{2+} uptake by endoplasmic reticulum Ca^{2+} -ATPase (32), did not abolish $[\text{Ca}^{2+}]_i$ oscillations in spontaneously active cells. As shown in Fig. 9A, upper panel, addition of thapsigargin increased the spiking frequency. In quiescent cells, thapsigargin increased $[\text{Ca}^{2+}]_i$ in a non-oscillatory manner, whereas Bay K 8644 was able to initiate $[\text{Ca}^{2+}]_i$ transients in the presence of thapsigargin (Fig. 9A, bottom tracing). Spontaneous (Fig. 9B, upper tracing) and Bay K 8644-induced $[\text{Ca}^{2+}]_i$ transients (Fig. 9B, bottom tracing) were also observed in cells exposed to 10 μM thapsigargin for 20 min prior to $[\text{Ca}^{2+}]_i$ recording, further indicating that the re-uptake of Ca^{2+} by endoplasmic reticulum is not required to generate $[\text{Ca}^{2+}]_i$ transients.

Second, the coupling of electrical activity to Ca^{2+} release by activating a voltage-sensor was ruled out in experiments with depolarization of somatotrophs bathed in Ca^{2+} -depleted medium. As shown in Fig. 9C, depolarization of cells did not affect base-line $[\text{Ca}^{2+}]_i$ after the extracellular Ca^{2+} was adjusted to about 100 nM. When cells were bathed in 0.25 and 0.5 mM Ca^{2+} -containing medium, a non-oscillatory rise in $[\text{Ca}^{2+}]_i$ was observed, with the amplitude of responses lower than those

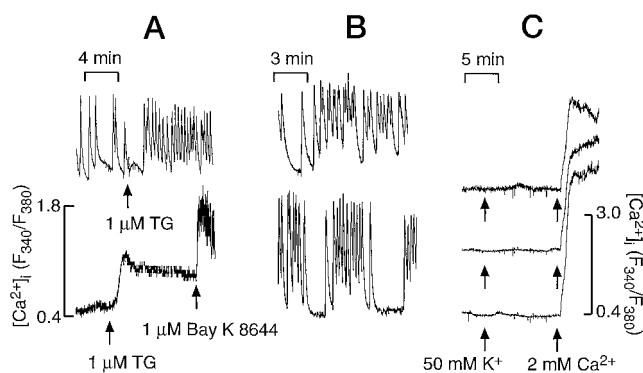


FIG. 9. Effects of thapsigargin and high potassium concentrations on $[\text{Ca}^{2+}]_i$ in cultured somatotrophs. A, effects of thapsigargin, a blocker of endoplasmic reticulum Ca^{2+} -ATPase, on $[\text{Ca}^{2+}]_i$ in spontaneously active (upper tracing) and quiescent cells (bottom tracing). Note the ability of Bay K 8644 to initiate spiking in the presence of thapsigargin. B, pattern of spontaneous $[\text{Ca}^{2+}]_i$ transients in somatotrophs treated with 10 μM thapsigargin for 20 min (upper tracings) and Bay K 8644-induced $[\text{Ca}^{2+}]_i$ transients (bottom tracing). C, high potassium concentration-induced depolarization. Somatotrophs bathed in Ca^{2+} -deficient medium (free $[\text{Ca}^{2+}]_o$ about 100 nM) were depolarized by 50 mM potassium.

observed in physiological extracellular Ca^{2+} concentrations (not shown).

Third, the participation of RyRs in spontaneous and Bay K 8644-induced $[\text{Ca}^{2+}]_i$ transients was excluded in experiments with caffeine and ryanodine, the modulators of these channels (33). As shown in Fig. 10A, caffeine was unable to initiate $[\text{Ca}^{2+}]_i$ transients in quiescent cells (upper tracing) or to change the rhythm of Ca^{2+} spiking in spontaneously active cells (two bottom tracings). Ryanodine, at its low stimulatory (5 μM) concentration, was also unable to initiate $[\text{Ca}^{2+}]_i$ transients (Fig. 10B, upper tracing) or to change the pattern of spiking in spontaneously active cells (two bottom tracings). This compound was also ineffective at its high inhibitory (100 μM) concentrations (Fig. 10C).

Cationic Channels and Spontaneous $[\text{Ca}^{2+}]_i$ Transients—Addition of TTX, a specific blocker of voltage-gated Na^+ channels, did not affect the rhythm of the spontaneous $[\text{Ca}^{2+}]_i$ transients (Fig. 11A, top three tracings). Also, in the presence of TTX, Bay K 8644 still modulated spontaneous $[\text{Ca}^{2+}]_i$ transients (Fig. 11A, the 2nd and 3rd tracings) and initiated oscillations in quiescent cells (Fig. 11A, two bottom tracings). However, in spontaneously active cells, $[\text{Ca}^{2+}]_i$ transients were immediately abolished by the removal of Na^+ , suggesting that the influx of this cation through TTX-insensitive channels is involved in initiating Ca^{2+} spiking. In a majority of cells, inhibition was transient and was followed by recovery of Ca^{2+} spiking after 5–15 min of exposure to Na^+ -deficient medium (Fig. 11B).

Elevation of extracellular $[\text{Ca}^{2+}]_o$ from 2 to 10 mM also abolished spontaneous $[\text{Ca}^{2+}]_i$ transients in a majority of the cells. Some cells responded to high extracellular Ca^{2+} with a reduction in the amplitude and frequency of $[\text{Ca}^{2+}]_i$ transients (Fig. 12A, top tracing), whereas in quiescent cells no changes in basal $[\text{Ca}^{2+}]_i$ were observed (not shown). Similarly, 10 mM Mg^{2+} (Fig. 12B) and Sr^{2+} (Fig. 12C) abolished spiking or reduced the frequency and/or amplitude of spontaneous $[\text{Ca}^{2+}]_i$ transients, suggesting the sensitivity of these Na^+ -conducting channels to high concentrations of divalent cations. Such a cationic profile is consistent with the presence of CNGs and their coupling to Ca^{2+} signaling pathway in somatotrophs (34).

To address this hypothesis, RT-PCR analysis was performed, using mRNAs from mixed population of anterior pituitary cells and purified somatotrophs and specific primers for three rat

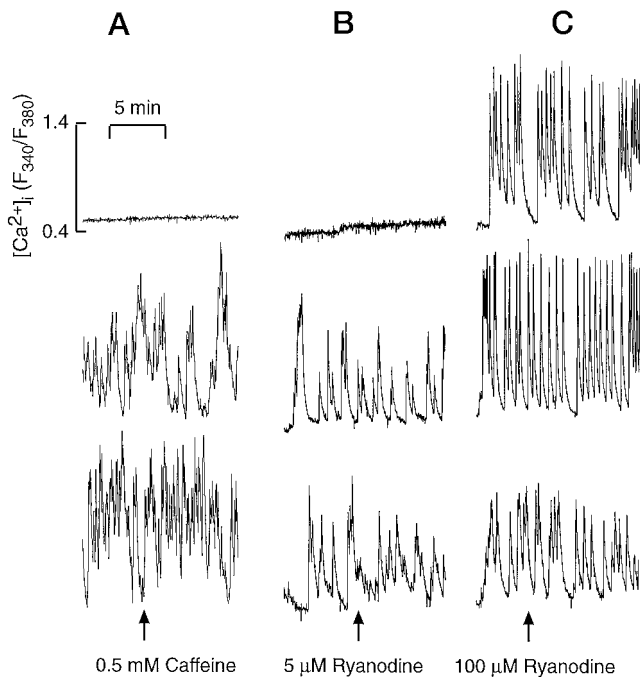


FIG. 10. Calcium influx is not coupled to calcium-induced calcium release in somatotrophs. A, the lack of effects of caffeine, an activator of ryanodine receptor-channels, on the pattern of spontaneous $[\text{Ca}^{2+}]_i$ transients. B and C, the lack of effects of a stimulatory (B) and inhibitory (C) concentrations of ryanodine on $[\text{Ca}^{2+}]_i$ in somatotrophs. Bay K 8644 ($1 \mu\text{M}$)-induced $[\text{Ca}^{2+}]_i$ transients are shown in C, two upper tracings. The arrows at the bottom indicate the moments of drug application in all tracings shown above.

channels: rod, cone, and olfactory. As in $[\text{Ca}^{2+}]_i$ measurements, dispersed cells were cultured for 24 h prior to RNA isolation. In mixed pituitary cells, the expected size of PCR products for all three CNGs was detected (Fig. 13). Moreover, in purified somatotrophs, only the specific signal for rod type channel was observed (Fig. 13, upper panels). Southern blot analysis of the same gels further confirmed these results (Fig. 13, bottom panels). No PCR products were detected from controls containing all the components except for reverse transcriptase (RT), ruling out the possibility of genomic DNA contamination.

GHRH-controlled $[\text{Ca}^{2+}]_i$ Signals—In further experiments, cells were stimulated with GHRH, a well established agonist for somatotrophs (27, 35, 36). Addition of 1 nM GHRH initiated Ca^{2+} spiking in quiescent cells (Fig. 14A, bottom tracing). In spontaneously active cells, 1 nM GHRH increased frequency of $[\text{Ca}^{2+}]_i$ transients (Fig. 14A, two middle tracings) or induced a sustained non-oscillatory elevation in $[\text{Ca}^{2+}]_i$ (Fig. 14A, two upper tracings). The majority of cells stimulated with 100 nM GHRH responded with a non-oscillatory increase in $[\text{Ca}^{2+}]_i$ that lasted as long as the agonist was present in the medium (up to 20 min). GHRH action on Ca^{2+} signaling was accompanied by a dose-dependent increase in cAMP production measured in the absence of phosphodiesterases, leading to a 50-fold increase in cAMP levels when cells were stimulated with 100 nM GHRH. In addition to cAMP, GHRH also stimulated cGMP production in a dose-dependent manner but with a shift in the EC_{50} of about 1 log unit (Fig. 14B).

DISCUSSION

Oscillations in intracellular calcium concentrations ($[\text{Ca}^{2+}]_i$ transients) can result from inositol trisphosphate-induced release of intracellularly stored Ca^{2+} and/or from voltage-gated and voltage-insensitive Ca^{2+} influxes. The first type of $[\text{Ca}^{2+}]_i$ transients is well characterized in a number of non-excitable and excitable cells, including hepatocytes, acinar cells, oocytes,

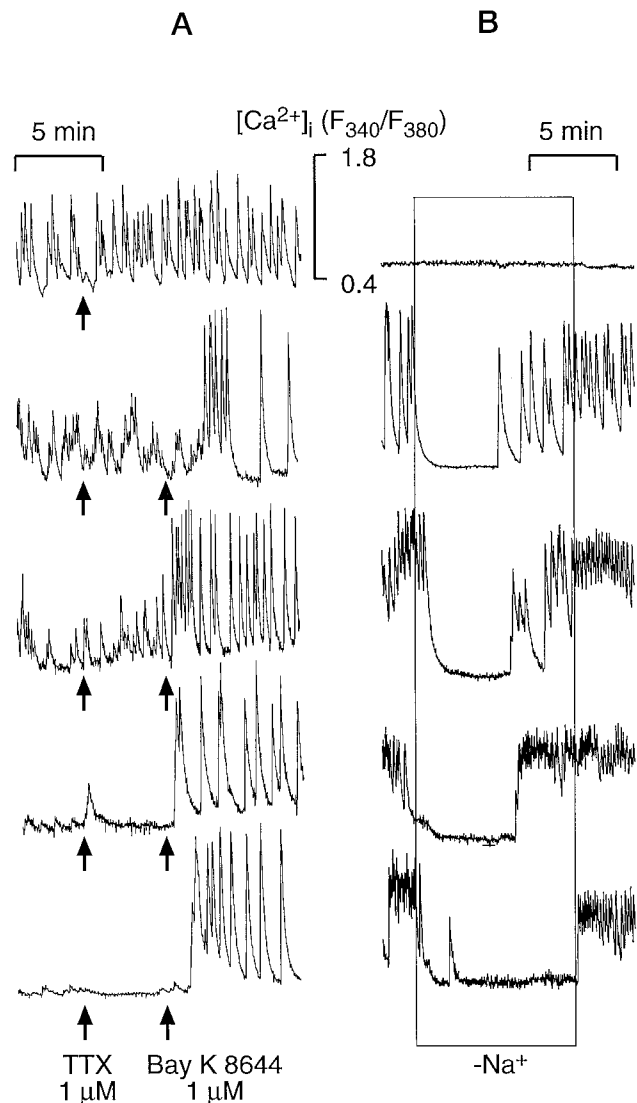


FIG. 11. Sodium dependence of Ca^{2+} transients in cultured somatotrophs. A, the lack of effects of tetrodotoxin (TTX), a blocker of voltage-gated Na^+ channels, on spontaneous and Bay K 8644-induced $[\text{Ca}^{2+}]_i$ oscillations. B, transient abolition of Ca^{2+} spiking in cells perfused with Na^+ -deficient medium (NMDG was used to substitute for Na^+). The rectangle indicates the time when cells were perfused with NMDG-containing medium.

and pituitary gonadotrophs (23, 37–40). Calcium-mobilizing agonist-induced $[\text{Ca}^{2+}]_i$ oscillations do not occur synchronously within the cell but originate from a specific sub-plasma membrane locus and propagate throughout the cell (41), including the nucleoplasm. The equilibrium between cytoplasmic and nuclear $[\text{Ca}^{2+}]$ is probably achieved by the release of Ca^{2+} from nuclear envelope in synchrony with its release from endoplasmic reticulum (42–44).

In cells exhibiting spontaneous- or receptor-controlled firing of APs, changes in plasma membrane potential can trigger Ca^{2+} influx through VGCCs. Since the activation of these channels is limited by the duration of depolarization, AP-driven Ca^{2+} signals are usually localized events that are relevant in the control of plasma membrane-associated cellular functions, such as excitability and neurotransmission (11). However, in cells expressing RyRs these localized signals may also trigger CICR from intracellular stores, leading to the formation of global Ca^{2+} signals (17, 18). Thus, both calcium-mobilizing receptors and APs can generate local and global Ca^{2+} signals and propagation of Ca^{2+} signals throughout the cell is coupled

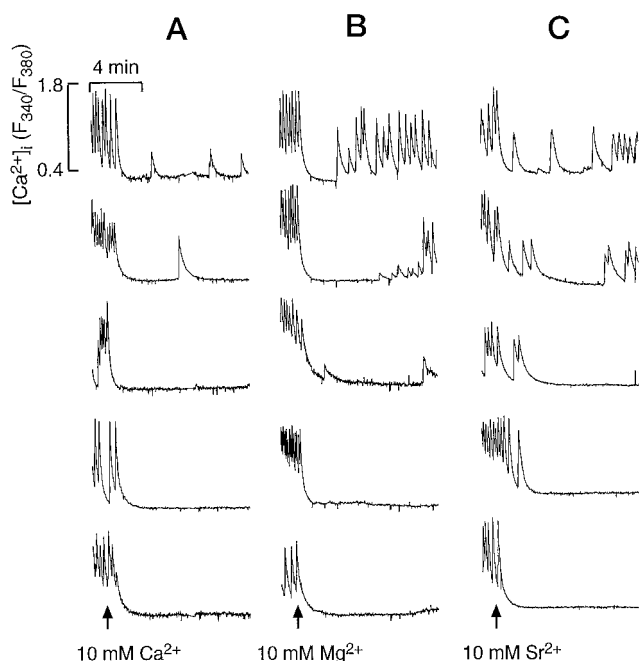


FIG. 12. **Inhibition of $[\text{Ca}^{2+}]_i$ transients by divalent cations.** A–C, effects of 10 mM extracellular Ca^{2+} (A), Mg^{2+} (B), and Sr^{2+} (C) on Ca^{2+} spiking. Addition of 10 mM NaCl to bath medium did not alter the pattern of $[\text{Ca}^{2+}]_i$ transients (not shown). The arrows at the bottom indicate the moments of drug application in all tracings shown above.

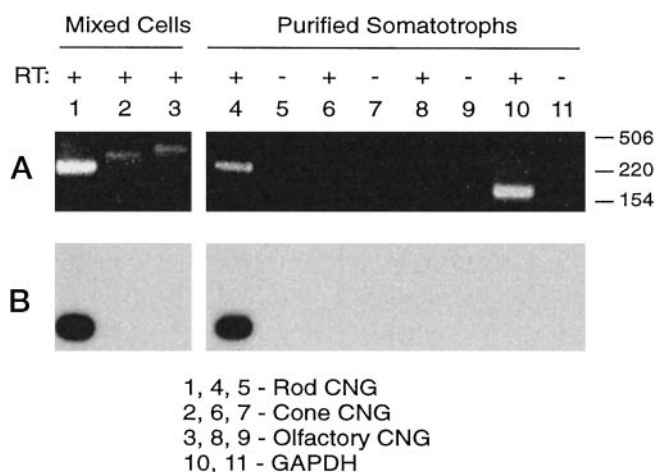


FIG. 13. **Detection of CNG transcripts in mixed anterior pituitary and purified somatotrophs.** A, RT-PCR analysis for rod (210 base pairs), cone (281 base pairs), and olfactory (318 base pairs) CNGs in mixed pituitary cells and purified somatotrophs. B, Southern blot analysis of the same gels shown in upper panels. Blots were probed with 5'-end-labeled oligonucleotide specific to the rod type CNGs.

to release of Ca^{2+} from intracellular stores.

Here we show that somatotrophs are able to generate spontaneous $[\text{Ca}^{2+}]_i$ transients, with single spikes lasting for several seconds. The elevations in $[\text{Ca}^{2+}]_i$ were detected in all regions of the cytoplasm, as well as in the nucleoplasm, indicating the global nature of $[\text{Ca}^{2+}]_i$ transients. In the non-normalized images, Ca^{2+} signal appeared to initiate in nucleoplasm and to terminate in cytoplasm. However, the extended time scale analysis of F/F_0 profiles revealed that the rise in $[\text{Ca}^{2+}]_i$ occurred almost simultaneously in cytoplasmic and nucleoplasmic regions. Also, $[\text{Ca}^{2+}]_i$ profiles in all monitored cell regions were highly comparable, suggesting that they were driven by the same mechanism. The apparent amplitudes of the Ca^{2+} signals, expressed as F/F_0 were similar throughout the cytoplasm, including the sub-plasma membrane region,

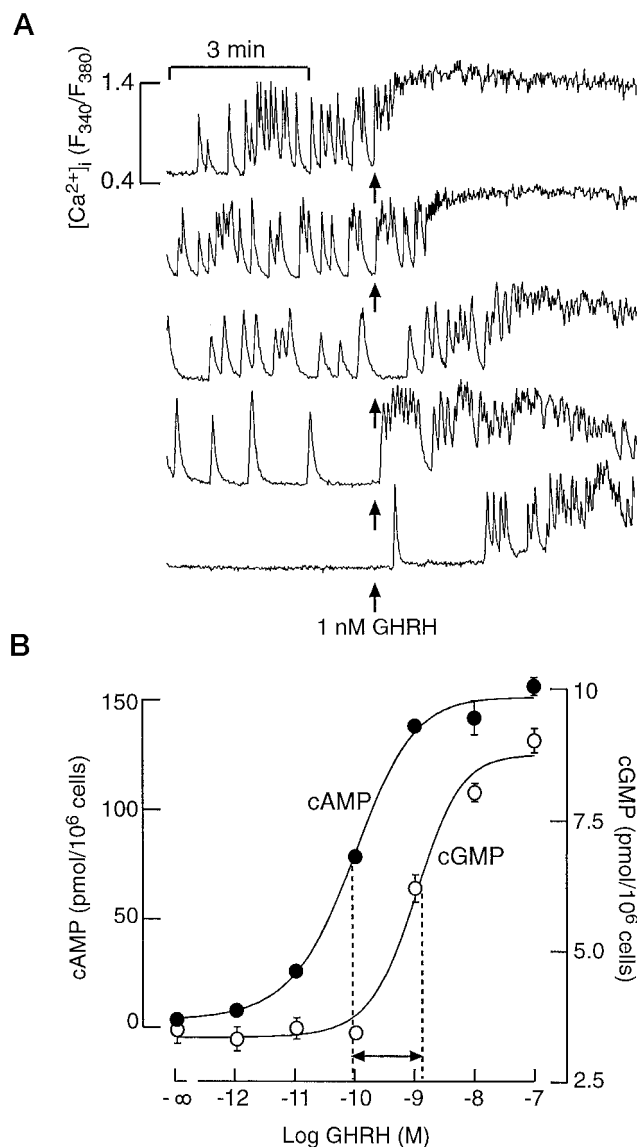


FIG. 14. **Effects of growth hormone-releasing hormone (GHRH) on $[\text{Ca}^{2+}]_i$ signaling and cyclic nucleotide production in pituitary somatotrophs.** A, stimulatory effects of GHRH on $[\text{Ca}^{2+}]_i$ in active (upper tracings) and quiescent cells (bottom tracing). B, dose-dependent effects of GHRH on cAMP and cGMP production in pituitary cells. The results shown are mean \pm S.E. from sextuplicate determination. The dotted lines indicate the EC_{50} values derived from fitted logistic curves. Cells were stimulated for 30 min with GHRH in the absence of phosphodiesterase inhibitors.

and were higher in the nucleoplasm. These differences should be taken with reservation, because of the controversies in comparing signals originating from different cellular compartments (43–45). In addition, the lack of difference in the amplitude of $[\text{Ca}^{2+}]_i$ responses close to the plasma membrane and in the central cytoplasmic regions does not rule out the existence of highly localized $[\text{Ca}^{2+}]_i$ elevations in sub-plasma membrane regions, since the resolution of our measurements may not be sufficient to detect such changes.

High amplitude spontaneous $[\text{Ca}^{2+}]_i$ transients are not unique for somatotrophs, as immortalized pituitary cells also exhibit such changes (1–10). The role of voltage-gated Ca^{2+} influx in spontaneous electrical activity and $[\text{Ca}^{2+}]_i$ transients in these cells is well established (11). Several reports have also indicated the expression of RyRs and their coupling to spontaneous electrical activity (7, 14, 15, 46, 47). The presence of specific mRNA for RyRs in a mixed population of pituitary cells

(46) is consistent with the coupling of voltage-gated Ca^{2+} influx to CICR in native pituitary cells as well. In further parallelism with immortalized cells, spontaneous $[\text{Ca}^{2+}]_i$ transients in somatotrophs were affected by high concentration of dihydropyridines, Cd^{2+} , and Ni^{2+} . Others have reported about the expression of VGCCs and participation of voltage-gated Ca^{2+} influx in spontaneous and GHRH-controlled electrical activity in somatotrophs (19) and GC somatotroph lines (3).

However, the present data indicate that spontaneous electrical activity in somatotrophs is not coupled to Ca^{2+} release from intracellular stores. The lack of CICR in somatotrophs was documented by the inability of two modulators of RyRs, caffeine and ryanodine (33), to initiate $[\text{Ca}^{2+}]_i$ transients in quiescent cells and/or to alter the pattern of signaling in spontaneously active cells. Also, spontaneous $[\text{Ca}^{2+}]_i$ transients were observed in cells treated with thapsigargin and ionomycin, showing that the plasma membrane oscillator is operative even upon the depletion of intracellular Ca^{2+} pools. Finally, in contrast to hippocampal neurons (48), depolarization of somatotrophs bathed in Ca^{2+} -deficient medium did not generate Ca^{2+} signaling, arguing against the operation of a voltage sensor in these cells. Thus, voltage-gated Ca^{2+} influx is able to generate high amplitude $[\text{Ca}^{2+}]_i$ oscillations without the integration of a Ca^{2+} release mechanism.

The dependence of spontaneous $[\text{Ca}^{2+}]_i$ transients on voltage-gated Ca^{2+} influx and their independence on Ca^{2+} release from intracellular stores implies a prolonged opening of VGCCs, presumably by a sustained activation of a depolarizing current needed to oppose the K^+ channel-mediated repolarization of the cells. The expression of depolarizing T-type Ca^{2+} and TTX-sensitive Na^+ channels in somatotrophs (49, 50) could provide a transient but not a prolonged depolarization of cells, because of their rapid and voltage-dependent inactivation (51). Earlier studies have indicated the TTX-insensitive Na^+ dependence of spontaneous $[\text{Ca}^{2+}]_i$ oscillations in GH tumor somatotrophs (3). Also, GHRH-induced electrical activity and Ca^{2+} signaling are dependent on extracellular Na^+ (20, 35, 52). In parallel to this, here we show that a substitution of Na^+ with NMDG leads to a temporal suppression of spontaneous $[\text{Ca}^{2+}]_i$ oscillations in cultured somatotrophs. The transient nature of inhibition of spontaneous $[\text{Ca}^{2+}]_i$ oscillations by NMDG further suggests that this compound or other ion(s) can substitute for Na^+ . That is consistent with the role of a non-selective ion channel in generating spontaneous $[\text{Ca}^{2+}]_i$ oscillations.

A number of channel subtypes are non-selective and can generate the pacemaking depolarizing currents, including CNGs that conduct Na^+ , Ca^{2+} , and K^+ (34). Three lines of evidence support the hypothesis that the rod type of CNGs function as non-selective cationic channels in spontaneously active somatotrophs. First, $[\text{Ca}^{2+}]_i$ transients in somatotrophs are inhibited by divalent cations in concentrations that inhibit CNGs as well. Second, the specific PCR product of rod type CNGs was obtained from highly purified somatotrophs. Third, GHRH initiated spiking in quiescent cells and increased the frequency of oscillations in spontaneously active somatotrophs, as well as increased the production of two messengers needed for the activation of CNG channels, cAMP and cGMP. However, to confirm the role of CNGs in the formation of $[\text{Ca}^{2+}]_i$ transients, additional experiments are required, including electrophysiological characterization of CNG current, the use of specific intracellular blockers, such as L-Cys-diltiazem, and Western blot analysis.

In conclusion, the presented results demonstrate that a plasma membrane Ca^{2+} oscillator is operative in somatotrophs and is capable of generating spontaneous high amplitude Ca^{2+} signals. Although there is no coupling of electrical activity to

intracellular Ca^{2+} release, this oscillator induces global Ca^{2+} signals. The results further suggest the physiological relevance of the spontaneous activity in the control of basal GH secretion. The operation of plasma membrane Ca^{2+} oscillator in somatotrophs is dependent on TTX-insensitive Na^+ conductance and voltage-gated Ca^{2+} influx. The oscillatory activity was inhibited by a high concentration of divalent cations, indicating a role of CNGs in pacemaker activity. Furthermore, the specific messengers for these channels were identified in pituitary cells. In agreement with the hypothesis that cyclic nucleotides are intracellular messengers controlling the plasma membrane Ca^{2+} oscillator activity, GHRH, an agonist that signals through adenylyl and guanlylyl cyclase pathways, initiated $[\text{Ca}^{2+}]_i$ spiking in quiescent cells and increased the frequency of spiking in spontaneously active cells.

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